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**Introduction:**

The signal transduction specificity that underlies Epidermal Growth Factor (EGF) Receptor (EGFR) physiology is an important component of tissue growth and development . Identifying the basic molecular mechanisms that regulate this signaling network is an important step in distinguishing the difference between positive (normal growth) and negative (uncontrolled cell proliferation) EGFR cell biology. A well described phenomena that accompanies EGFR signaling is the entry of the activated EGFR into the cell via clathrin coated vesicles (1). Inhibition of EGFR internalization results in the selective inhibition of some, but not all, signaling pathways (2-5). The purpose of this research is to further explore the spatial and temporal components of EGFR signal transduction by examining the signaling properties of the activated EGFR when it is trapped in an early endosomal vesicle. The scope of this research includes developing a model system to examine early endosomal signaling, assessing the biochemical properties of EGFR, and determining the signaling properties of the endosomally localized EGFR as well as those of other members of the EGFR family, namely ErbB2.

**Body:**

Growth Factors and their corresponding cell surface receptors are important components in the maintenance and growth of normal epithelial cells. Overexpression and unregulated signaling of members of the ErbB growth factor receptor family is associated with many mammary carcinomas and a poor prognosis for recovery (6,7). This proposed research seeks to better understand the coordinated regulation of signaling by the ErbB family of cell surface receptors, and more specifically, ErbB1 or the Epidermal Growth Factor (EGF) Receptor (EGFR). The EGFR, like many cell surface receptors, undergoes ligand mediated endocytosis. This internalization process has historically been thought to be merely a mechanism for attenuating receptor signaling by removing the activated receptor and ligand from the cell surface. It is now appreciated that the role of the endocytic pathways plays a more complex role in EGFR signaling (2-5). Our approach utilizes the introduction of mutant forms of proteins involved in membrane trafficking. It is expected that overexpression of these proteins will disrupt normal endocytic trafficking of the EGFR allowing for analysis of EGFR signaling at distinct cellular locations.

Rab5 is a small molecular weight GTP-binding protein that has been well described as an important regulator in the formation of the early endosome (8,9). A point mutation of rab5 (glutamine to lysine mutation at residue 79 – denoted Q79L) reduces the ability of the to hydrolyze GTP thus, leaving the protein in the constitutively active state. The consequence of this mutation is an enlarged early endosome enriched in transferrin receptors (9,10). Our model system incorporated HeLa cells stably transfected with wild type (WT) and activated mutant (Q79L) rab5 under a tetracycline regulated system.

Specifically, in the induced rab5 (Q79L) HeLa cells we observed the characteristic enlarged endosomal morphology as well as immunofluorescent localization of rab5, transferrin and transferrin receptors. These data indicated that we had successfully recapitulated our desired model system. When the endocytic trafficking of the EGFR was examined, quite surprisingly, we discovered there was no change in the rate of EGFR uptake, degradation or recycling.

As experiments progressed and the rab5 (Q79L) HeLa cells were subjected to a more rigorous biochemical characterization, we discovered that in our stably transfected HeLa cells the endosomal morphology and cellular localization of rab5 alone did not accurately predict changes in the kinetics of transferrin receptor endocytosis and/or recycling. We postulated that the levels of rab5 expression were not sufficient to give the desired biochemical effect. To eliminate this problem, we generated and characterized tetracycline inducible adenoviruses that encoded for both the WT and Q79L rab5 proteins. This new model system allowed rapid and uniform introduction of high levels of the rab5 proteins. We began our studies with the morphological and immunofluorescent characterization of the cells. Once again, these data were consistent with those reported in the literature, however upon biochemical analysis of transferrin receptor uptake and recycling, we failed to see the reported phenotype.

A second explanation for the inability to see the expected phenotype was that the cells had compensated for the rab5(Q79L) overexpression, allowing the EGFR receptor to utilize other mechanisms for membrane trafficking. To test this hypothesis, we developed a strategy to rapidly express the rab5 proteins at high levels (approximately 50-fold over endogenous protein) and in a relatively short period of time (1 hour).

Analysis of rab5 expression under these conditions yield the same result: the morphological phenotype but no change in EGFR membrane trafficking.

At this point, we began to suspect that the originally reported data were misinterpreted. Initial publications suggested that rab5 played a role in coordinating the membrane trafficking of the transferrin receptor by regulating fusion to the early endosomal compartment. Thus, when a constitutively activated mutant of rab5 is present, transferrin receptor endocytosis increases, receptor recycling decreases, and the early endosome becomes enlarged. While we were able to see the enlarged endosomes, we did not see the accompanying changes in endosomal trafficking; therefore, the enlarged endosomes were not sufficient to predict biochemical changes in membrane trafficking. In a through analysis of our results, we confirmed that the enlarged endosomes appropriately localized with various markers of cellular compartments (i.e. EEA1, rab7, rab9, LAMP-1, mannose-6- phosphate receptor, and the trans golgi network). The enlarged endosomes could effectively traffic the transferrin receptor through them as demonstrated by the appearance and disappearance of Alexa-Transferrin in them. Kinetic examination of markers of membrane trafficking – uptake and recycling of transferrin, fluid phase uptake (a non-specific marker of membrane trafficking), and lipid recycling (a non-specific marker of membrane recycling).

From these experiments, we have concluded that while rab5(Q79L) can induce the formation of an enlarged early endosome, this endosome is not an accurate predictor of changes in the kinetics of transferrin receptor uptake and/or recycling. These data are being written up and will be submitted as a manuscript to The Proceedings of the National Academy of Science (U.S.A.).



The above described work has provided a “hands on” approach to learning membrane trafficking. As indicated in my proposal, I joined the laboratory of Dr. Sandra Schmid to learn the science of membrane trafficking to complement my existing expertise receptor tyrosine kinase signal transduction. Although these results were unexpected, the rigorous analysis of membrane trafficking has been an excellent training. With this project under my belt as well as the co-authoring a review for Current Opinions in Cell Biology (11), I am very well prepared to study the second and third specific aims.

## **Appendix to Summary:**

### **1) Key Research Accomplishments:**

- Characterization of stable rab5(WT) and rab5(Q79L) HeLa celllines.
- Generation of tetracycline regulatable adenoviruses encoding for rab5(WT) and rab5(Q79L).
- Demonstration that the rab5(Q79) formed enlarged endosome is retains its functionality
- Demonstration that the rab5(Q79) formed enlarged endosome is not an accurate predictor of changes in membrane trafficking.

## **2) Reportable Outcomes**

### **Manuscripts:**

- B. P. Ceresa and S. L. Schmid, Regulation of signal transduction by endocytosis (2000),  
Current Opinion in Cell Biology Vol 12 (2) p. 204-210. (Appendix A)
- B. P. Ceresa and S. L. Schmid, Endocytic trafficking occurs normally through  
rab5Q79L-induced enlarged endosomes. (Manuscript in preparation and to be  
submitted to PNAS).

### **Employment:**

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11. Ceresa, B. P., and Schmid, S. L. (2000) *Curr Opin Cell Biol* **12**, 204-210

## **Appendix A:**

### **Published Manuscripts:**

Ceresa, B. P., and Schmid, S. L. (2000) *Curr Opin Cell Biol* **12**, 204-210

# Regulation of signal transduction by endocytosis

Brian P Ceresa and Sandra L Schmid

Endocytosis of ligand-activated receptors has generally been considered a mechanism to attenuate signaling. There is now a growing body of evidence suggesting that this process is much more sophisticated and that endocytic membrane trafficking regulates both the intensity of signaling and the co-localization of activated receptors with downstream signaling molecules.

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## Abbreviations

<b>β<sub>2</sub>AR</b>	β <sub>2</sub> adrenergic receptor
<b>EBP50</b>	ERM-binding phosphoprotein-50
<b>EGF</b>	epidermal growth factor
<b>EGFR</b>	epidermal growth factor receptor
<b>ERK 1/2</b>	extracellular regulated kinases 1 and 2
<b>ERM</b>	ezrin–radixin–moesin
<b>G protein</b>	guanine-nucleotide-binding protein
<b>GPCR</b>	G-protein-coupled receptor
<b>IRS-1</b>	insulin receptor substrate-1
<b>NDF</b>	Neu differentiation factor
<b>PI3-K</b>	phosphatidylinositol 3-kinase
<b>PLD</b>	phospholipase D
<b>RTK</b>	receptor tyrosine kinase
<b>TGF-α</b>	transforming growth factor-α

## Introduction

Cell surface receptors are the molecules through which changes in the extracellular environment are communicated within the cell. Among the diverse cellular responses to ligand-mediated signaling events are the uptake of nutrients and ions, the regulation of protein and DNA synthesis, and decisions about the proliferation or death of the cell. These responses are triggered when intracellular signaling molecules are activated or generated through signaling pathways initiated by ligand-bound cell surface receptors. However, it remains poorly understood how these resultant signaling pathways are regulated to form a co-ordinated, receptor-specific response. A cell can express a variety of cell surface receptors, which utilize a limited number of directed and regulated signaling pathways; yet each receptor produces a distinct response in cell physiology. The interactions of activated cell surface receptors with downstream effectors needed to amplify and transduce biochemical signals are governed by such diverse cellular processes as membrane trafficking, compartmentalization and regulated protein expression. This review focuses on the interplay between membrane trafficking and signaling by cell surface receptors. We discuss how membrane trafficking regulates signal transduction and how signaling events, in turn, regulate distinct steps in membrane trafficking.

## Caveolae as coordinators of signaling molecules

One well-studied example of compartmentalized signaling occurs from caveolae, which are morphologically defined as 'omega'-shaped invaginations of the plasma membrane. Biochemically, these membrane domains are defined by their association with a family of cholesterol-binding proteins called caveolins, which function to establish and/or maintain these structures. Caveolae constitute microdomains of the plasma membrane that are enriched in cholesterol, glycosphingolipids and lipid-anchored membrane proteins. With over 30 membrane receptors, signaling molecules and membrane transporters localized to caveolae, these lipid- and protein-dense cell surface microdomains are natural candidates for centers of signaling activity (reviewed in [1]).

Recent compelling evidence that caveolae are signaling centers comes from analysis of the direct consequences of modulating endogenous levels of caveolin or interfering with caveolin function in signaling. For instance, using an antisense strategy to inhibit the expression of caveolin-1 in NIH-3T3 cells causes their transformation by facilitating anchorage-independent growth and hyperactivation of extracellular regulated kinases 1 and 2 (ERK 1/2) [2]. Signaling through these pathways was restored when caveolin-1 returned to endogenous levels. Correspondingly, overexpression of caveolin-1 suppresses ERK 1/2 signaling [3]. Caveolin overexpression does not have an inhibitory effect on all signaling pathways. The expression of recombinant caveolin-1 in NIH-3T3 cells causes an increase in phospholipase D1 (PLD1) activity [4].

A more striking example of the specificity of caveolae-dependent signaling events is the finding that the disruption of caveolin function or caveolae structure, caused either by overexpression of dominant-negative caveolin-3 mutants or by depletion of cellular cholesterol, interferes with the activation of the protein kinase raf by activated H-ras without affecting its activation by the almost identical isoform K-ras [5]. The difference between these two ras isoforms is their localization at the plasma membrane: H-ras, but not K-ras, is palmitylated, a modification that targets it to the cholesterol-rich lipid microdomains associated with caveolin. A functional relationship between caveolin expression and cholesterol was also observed in *Caenorhabditis elegans*, in which either reduction of caveolin expression by RNA interference or depletion of cholesterol perturbs ras signaling through the MAP kinase pathway [6].

The dramatic and diverse effects that caveolin function has on regulating signaling molecules are probably the result of using extreme measures to manipulate a system

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Recent data suggest that even the release of Hh may be regulated by cholesterol. A gene called *dispatched* was identified in a screen for mutations with a Hedgehog-like phenotype. Like patched, it has sterol-sensing domain but seems to be required for the release of Hh from the cells that synthesize it. Intriguingly, dispatched does not facilitate the release of GPI-anchored Hh, suggesting that the release of normal cholesterol-modified Hh is not simply related to raft association.

that is, under normal conditions, more delicately balanced by much smaller changes in caveolin function. In NIH-3T3 and Rat1 cells, mutationally activated c-erbB2 (c-neu) causes the downregulation of caveolin-1, but not caveolin-2 [7]. These decidedly more subtle changes are probably part of a complex feedback mechanism to regulate transformation potential under prolonged growth factor stimulation.

The exact role of caveolin and caveolae remains enigmatic. There is good evidence that they play direct roles in regulating plasma membrane cholesterol levels [8]. Although caveolae are clearly regions enriched with signaling proteins, it remains uncertain whether these structures function to spatially coordinate signaling events or whether there exists a more direct role for cholesterol and membrane subdomain composition itself in controlling the activity of signaling complexes.

### Endocytosis as a regulator of signal transduction

Ligand-mediated endocytosis is characteristically an early response in the signaling pathways triggered by a diverse group of cell surface receptors, including heterotrimeric guanine-nucleotide-binding protein (G protein)-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and cytokine receptors [9,10]. Upon binding of the ligand (hormone, neuropeptide, growth factor, odorant, etc.) to its cognate cell surface signaling receptor, the activated receptors are targeted to clathrin-coated membrane invaginations, which, through a series of highly regulated, yet still not fully understood, biochemical events, eventually pinch off to form a clathrin-coated vesicle. Subsequent membrane fusion and budding reactions deliver the contents of the vesicle through sequential endosomal compartments. During progression along the endocytic pathway, the endosomes are modified in protein composition and pH, and their contents are sorted for shipment to the appropriate cellular destination. Among these fates are retention in the endosomal compartment, recycling back to the plasma membrane and delivery toward a lysosomal degradation pathway.

Ligand-mediated endocytosis plays at least two functions in receptor signaling. First, it can serve as a biophysical mechanism for attenuating the signaling of an activated cell surface receptor. Discussed in this review is the evolving story of the ErbB RTK family, which illustrates how controlled receptor trafficking regulates the potency of mitogenic signaling. Second, endocytosis plays a role in placing the activated cell surface receptor in the appropriate cellular location to interact with downstream signaling molecules. Signaling to ERK 1/2 by the internalized  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) and the epidermal growth factor (EGF) receptor (EGFR) not only demonstrates this phenomena but also provides insight into how these interactions might be regulated.

### Endocytosis as a means of regulating receptor activity

An excellent example of how endocytic membrane trafficking can regulate signaling comes from studies on the ErbB family of RTKs. ErbB family members, including the EGFR (ErbB1), are activated upon dimerization induced by binding their ligands, which are EGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ) and Neu differentiation factor (NDF). The specific ligand determines the composition of the dimeric receptor (Figure 1).

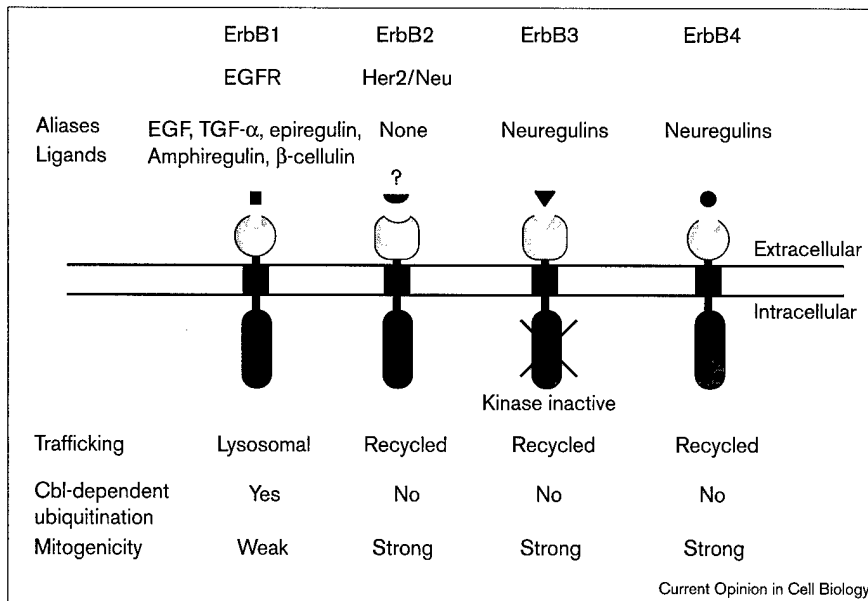
It has been appreciated for some time that different ErbB receptor ligands invoke different signaling potencies, particularly in terms of their mitogenic potential [11]. This has been convincingly demonstrated and mechanistically explored by stably transfecting cells lacking ErbB family members with ErbB1 alone or in combination with either ErbB2 or ErbB3 [12\*]. Cells expressing only ErbB1 were less proliferative in response to EGF than in response to TGF- $\alpha$ . When either ErbB2 or ErbB3 was co-expressed with ErbB1, the cells became significantly more responsive to EGF, without altering their response to TGF- $\alpha$ . These increases in mitogenic response correlate with the increased recycling and decreased downregulation of ErbB1 homodimers that occur in the presence of TGF- $\alpha$  or when ErbB2- or ErbB3-containing heterodimers are activated by either EGF or TGF- $\alpha$ .

As implied by these results, endosome sorting is regulated by interaction with both luminal and cytoplasmic domains of the ErbB family members. Two lines of evidence suggest that endosome acidification and the pH-dependent dissociation of receptor-ligand complexes are central in controlling this sorting decision. First, TGF- $\alpha$  and NDF dissociate from their receptors at a relatively higher pH than EGF, suggesting that this dissociation would occur in early endosomal compartments, which are involved in receptor recycling. Second, treating cells with the ionophore monensin, which increases the pH of endosomal sorting compartments, leads to increased downregulation of receptors activated with TGF- $\alpha$  and NDF [12\*,13\*]. Studies with chimaeric receptors encoding the ligand-binding extracellular domain of ErbB1 and the intracellular domain of ErbB2 [14] or other ErbB family members [15] indicate that sorting signals in the ErbB1 cytoplasmic domain are required for receptor degradation.

One candidate molecule that may recognize sorting determinants in ErbB1 is c-Cbl or its *C. elegans* ortholog Sli-1. c-Cbl has been shown to be a downstream substrate and negative regulator of a number of cell surface receptors, although its mechanism has been poorly understood [16,17]. Levkowitz *et al.* [18] found that c-Cbl is recruited to endosomes in cells transiently expressing the lysosomally directed ErbB1, but not the recycling ErbB3. c-Cbl is required for ligand-dependent ubiquitination of ErbB1 in endosomes, a modification that may target the protein to the lysosome degradative pathway. Importantly, mutants of either the ErbB1 RTK or c-Cbl



Figure 1



Structural and signaling properties of the ErbB receptor tyrosine kinase family (see text for details).

that disrupt their interactions [18,19,20\*\*] result in decreased ErbB1 degradation and increased mitogenic signaling [19] (Figure 2a). Results using other cell surface signaling receptors suggest a general role for ubiquitination in regulating endocytic membrane trafficking [21].

### Pathophysiological consequences of receptor tyrosine kinase trafficking

The physiological significance of differential RTK trafficking has been demonstrated by studies utilizing cultured breast cancer cell lines expressing varying ratios of endogenous ErbB1 and ErbB2 RTKs. Through a series of biochemical and immunocytochemical studies, Wang *et al.* [22\*] demonstrate that, despite auto-tyrosine-phosphorylation of both family members upon EGF treatment, endocytosis of dimeric receptor complexes is inversely proportional to ErbB2 expression. The physiological consequence of the differential cellular trafficking is increased mitogenic signaling owing to the prolonged activation state of ligand–receptor complexes involving ErbB2 or ErbB3 subunits [12\*,13\*,23].

This selective degradation of only ErbB1 receptors may explain the more carcinogenic nature of other ErbB family members. For instance, expression of ErbB2 has long been correlated with many carcinomas and poor prognosis [24,25]. One plausible explanation for ErbB2's carcinogenic effect is its ability to increase the ratio of ErbB1–ErbB2 heterodimers over ErbB1 homodimers. In doing so, alterations in membrane trafficking would result in enhanced mitogenicity.

### Signaling from within the endosome

The concept that activated receptors interact with downstream signaling molecules at discrete endocytic locations

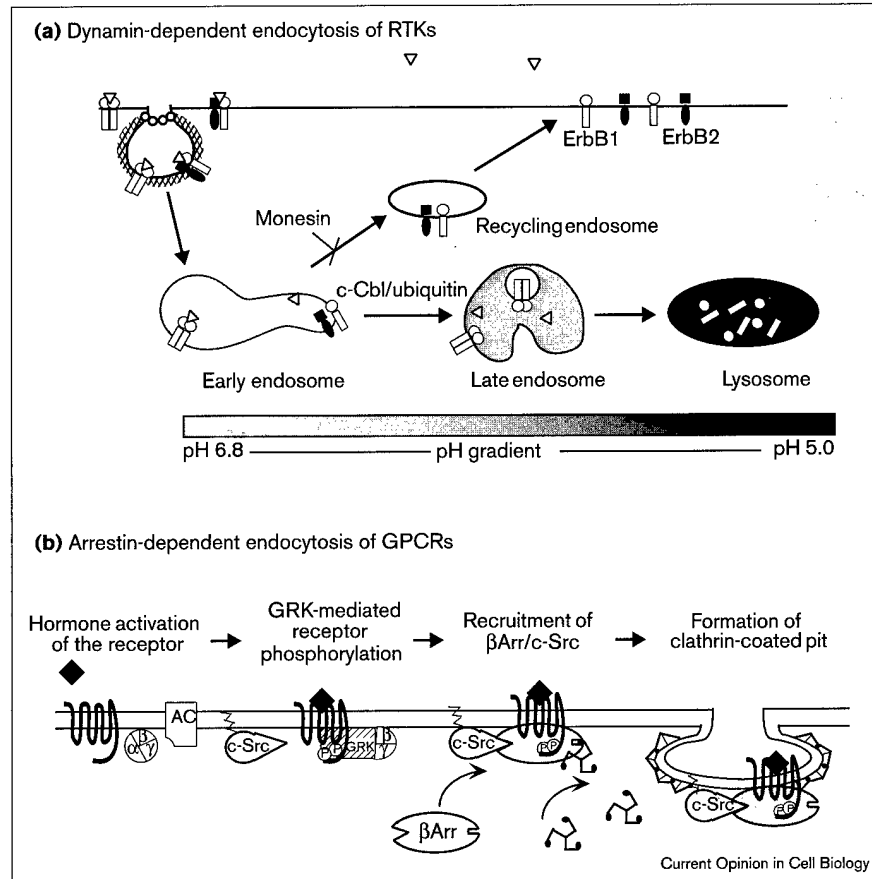
has been postulated for years [26,27]. Previously, testing this hypothesis was limited by the inability to trap an activated receptor at unique endocytic locales without significantly altering the receptor structure and/or impairing signaling pathways. Now, with a clearer understanding of the initial stages of endocytosis and receptor desensitization, less invasive cell biological methods for disrupting endocytosis have been developed. Thus, there has been a plethora of data examining receptor signaling prior to entry into the endocytic pathway.

The two most commonly used tools are dominant-negative constructs of the GTPase dynamin and the GPCR-binding protein arrestin. Overexpression of dominant inhibitory forms of dynamin (those that can not bind or hydrolyze GTP) blocks clathrin-mediated endocytosis [28], causing the retention of many, but not all, receptors at the cell surface. Overexpression of dominant-negative forms of arrestin [10,29\*] specifically blocks the endocytosis of GPCR. Arrestin binds activated GPCRs after they become phosphorylated on serine residues within their carboxyl termini through a GPCR-kinase-mediated feedback loop. Arrestin binding prevents activated GPCR further interacting with heterotrimeric G proteins and transducing signals. In addition, arrestins serve as adapter molecules that target activated GPCR to endocytic coated pits [30]. Together, these methods have proven effective in a direct comparison of the signaling of receptors retained on the cell surface with that of those allowed to enter the endocytic pathway.

Not surprisingly, there are some signaling pathways that are completely unaffected or enhanced by retaining activated receptors at the cell surface. These include the most proximal events in receptor signaling, such as the intramolecular kinase activity of RTKs, activation of heterotrimeric G proteins,

**Figure 2****Endocytosis of signaling receptors.**

**(a)** Differential endocytic trafficking of homodimeric and heterodimeric ErbB1 and ErbB2 RTKs. Ligand binding induces receptor dimerization and targeting to clathrin-coated pits, which pinch off in a dynamin-dependent manner. The coated vesicles form, then shed their clathrin coats and fuse with the mildly acidic early endosome, where the RTKs are sorted for recycling or degradation. Ligands dissociate from ErbB2-containing dimers, which are then sorted on a monensin-sensitive step to the recycling endosome and returned to the cell surface. ErbB1 dimers retain bound ligand until encountering the lower pH or later endosomes, where ErbB1 dimers associate with c-Cbl, are ubiquitinated and targeted for degradation in the lysosome. Within endosomes, ErbB1 dimers associate with c-Cbl, are ubiquitinated and targeted for degradation in the lysosome. **(b)** Formation of the  $\beta_2$ AR- $\beta$ -arrestin-c-Src complex as a mechanism to form the  $\beta_2$ AR-containing endosome. Upon hormone stimulation, the  $\alpha$  and  $\beta\gamma$  subunits of the activated heterotrimeric G protein dissociate to cause activation of downstream effectors such as adenylyl cyclase (AC). The  $\beta\gamma$  subunits facilitate the G-protein-coupled receptor kinase (GRK)-mediated phosphorylation of ligand-bound  $\beta_2$ AR.  $\beta$ -Arrestin ( $\beta$ Arr) binds both the phosphorylated  $\beta_2$ AR and c-Src, causing the formation of the  $\beta_2$ AR- $\beta$ -arrestin-c-Src complex at the plasma membrane. The  $\beta$ -arrestin interaction with clathrin targets the  $\beta_2$ AR to coated pits. Its subsequent internalization is a prerequisite for signaling to the ERK 1/2 pathway.



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phosphorylation of insulin receptor substrate-1 (IRS-1) by the insulin receptor, regulation of adenylyl cyclase activity and stimulation of phospholipase C $\gamma$ . Given the plasma membrane location and/or the kinetics of activation of these effectors, it seems unlikely that receptor endocytosis would be a prerequisite for their activity.

More intriguing are those events that are attenuated when receptor endocytosis is inhibited — namely the activation of phosphatidylinositol 3-kinase (PI3-K) and ERK 1/2 [31–36]. In the case of insulin receptor signaling, PI3-K associates with IRS-1 in an insulin-dependent manner as a prerequisite for signaling. When insulin receptor endocytosis is blocked by dominant-negative dynamin, there is a significant reduction in insulin-dependent PI3-K activity, despite full tyrosine phosphorylation of the insulin receptor and IRS-1. The change in kinase activity is reflected in a corresponding decrease in the amount of p85 regulatory subunit of PI3-K associated with IRS-1 [31].

Although there are a number of cell surface receptors that require endocytosis for ERK 1/2 activation, there are an equal number that do not (Table 1). One possible explanation is that the need for endocytosis is receptor-specific

and that the role of endocytosis in ERK 1/2 activation may be a mechanism through which signal specificity is conferred. These results are reminiscent of the differential effects on ras signaling through MAP kinase observed upon caveolae disruption (see above) and suggest that multiple mechanisms exist to spatially regulate the MAP kinase signaling pathway.

Interestingly, it has been reported that dominant inhibitory dynamin decreases  $\mu$  opiod receptor mediated activation of ERK 1/2 in HEK293 cells independent of an effect on the endocytosis of the  $\mu$  opiod receptor [37]. It is possible this is a consequence of dynamin's role in regulating an intermediate signaling protein whose endocytosis is required for ERK 1/2 activation. Alternatively, these data may suggest a second role for dynamin, in addition to regulating endocytosis. However, expression of dominant-negative dynamin has no effect on ERK 1/2 activation by the  $\alpha_2$  adrenergic receptor, which, like the  $\mu$  opiod receptor, couples to G $\alpha_{i/o}$  guanine-nucleotide-binding proteins and is internalized in a dynamin-independent manner [38].

Taken together, this selective inhibition of signaling pathways suggests that an activated receptor and requisite

Table 1

## Consequences of inhibited endocytosis on ERK activity.

Receptor	Endocytic block	Cell line	ERK activity	References
<b>Receptor tyrosine kinase</b>				
EGFR	Dynamin	HeLa	↓ 50%	[35]
IR	Dynamin	H4IIE	↓ 50%	[31]
<b>G-protein-coupled receptors</b>				
$\alpha_{2A}$ AR	Dynamin*	COS-1, HEK293	No change	[33,38]
$\alpha_{2B}$ AR	Dynamin	COS-1, HEK293	No change	[33,38]
$\alpha_{2C}$ AR	Dynamin	COS-1	No change	[33]
$\beta$ AR	Dynamin, arrestin	HEK293	↓ 60%	[32,45]
$\delta$ opioid	Dynamin	Cos-7, HEK293	↓ 80%	[46]
$\mu$ opioid	Dynamin	HEK293	↓ 80%	[46]
$\kappa$ opioid	Dynamin, arrestin	CHO	No change	[47]
	Dynamin	HEK293	↓ 80%	[46]
5HT <sub>1A</sub> Serotonin receptor	Dynamin, arrestin	HEK293	↓ 50%	[34]
LPA	Dynamin, arrestin	HEK293	↓ 50–80%	[32]
CXCR2	Dynamin	HEK293	No change	[48]

\*Endocytosis is dynamin independent.

signaling molecules are not sufficient to direct appropriate cellular responses — endocytosis is also an essential component.

### Signal transduction can regulate endocytosis

Although the role of the endocytic pathway in receptor signal transduction has only recently been appreciated, it has been known for some time that signaling receptors must be active for their endocytosis [39]. Our understanding of the role of receptor signaling in endocytosis has been limited to mutagenesis studies defining the receptor domains that are involved in recruitment to clathrin-coated pits and endocytosis. New studies using endocytosis-deficient cell lines have readdressed this mechanism and the nonreceptor tyrosine kinase c-Src has emerged as an important regulator of endocytosis.

The effect of the endocytic pathway on signaling and *vice versa* has been best characterized using  $\beta_2$ AR as a model. With the first observation of  $\beta_2$ AR internalization upon agonist stimulation came the hypothesis that this was strictly a mechanism by which activated receptors were removed from the cell surface and sequestered within the cell to prevent further activation of a downstream effector, namely adenylyl cyclase. This hypothesis continued to garner momentum with the observation of ligand-dependent phosphorylation and the subsequent association of  $\beta$ -arrestin to prevent further signaling to heterotrimeric G proteins.

More recent results have suggested an additional role for  $\beta$ -arrestin binding. In this work, Luttrell *et al.* [40•] demonstrate that agonist activation of  $\beta_2$ AR results in the formation of a  $\beta_2$ AR- $\beta$ -arrestin-c-Src complex. This protein complex

targets the receptor to a clathrin-coated pit (Figure 2b). It has been shown that kinase-inactive forms of c-Src can inhibit, and constitutively active forms of c-Src can enhance,  $\beta_2$ AR endocytosis [41]. Activation of both  $\beta_2$ AR and the LPA (lysophosphatidic acid) receptor leads to phosphorylation of dynamin, in the former case, through a c-Src-dependent process [41,42]. These data suggest that c-Src is an upstream regulator of dynamin function in  $\beta_2$ AR endocytosis. It has been shown that EGF-stimulated activation of c-Src leads to tyrosine phosphorylation of clathrin and that phosphorylation is required for the recruitment of clathrin to the membrane [43]. Taken together, these data strongly suggest a role for c-Src in receptor endocytosis — the question remains whether c-Src is a regulator of dynamin function or a recruiter of clathrin or both.

Another candidate protein for regulating  $\beta_2$ AR signaling is the ezrin-radixin-moesin (ERM)-binding phosphoprotein-50 (EBP50). EBP50 binds to the G-protein-regulated kinase 5 (GRK5)-phosphorylated cytoplasmic tail of  $\beta_2$ AR via a PDZ domain and to the cortical cytoskeleton through an ERM-binding domain. In HEK293 cells, the disruption of the  $\beta_2$ AR-EBP50-actin interaction results in diminished  $\beta_2$ AR recycling [44•]. Although EBP50 has not been shown to have a direct role in  $\beta_2$ AR signaling, its effects on  $\beta_2$ AR endocytic trafficking are likely to have important implications in signaling [44•], similar to those described above for ErbB family members.

### Conclusions

The key to fully understanding the cell physiology mediated by cell surface receptors lies not only in the identification of downstream effectors but also in the exquisite spatial and temporal regulation of the interactions

with these effectors. Clearly, membrane trafficking plays an important role both in controlling the location of signaling interactions and in regulating the cellular degradation and recycling of the activated receptor. From a biomedical prospective, the identification of the sites and knowledge of the kinetics of receptor activation of downstream effectors provides an opportunity to design rational therapeutic strategies to manipulate a given signaling pathway.

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